REMARKS

Entry of the foregoing, and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.116, are respectfully requested.

The Office Action Summary correctly indicates that claims 1-33 and 35-37 are pending in the application. Claims 4-16, 19, 23-33 and 35-36 have been withdrawn from consideration. Claims 1-3, 17, 18, 20-22 and 37 are under consideration and stand rejected.

By the present amendment, claim 1 has been amended. Claim 1 has been amended to recite that the polypeptide comprises "at least one amino acid sequence of at most 20 and at least 8 consecutive amino acids defined in SEQ ID NO: 1 . . ." Support for the amendment to claim 1 can be found throughout the specification, at least at page 7, lines 3-4.

Claim 17 has been amended to recite the claimed subject matter in another way. Support for claim 17, as amended, can be found at least in the claims as originally filed.

By the present amendment, claims 38 to 56, which recite "consisting essentially of," are added. The subject matter of claims 38 to 56 is supported throughout the specification and the original claims.

Applicants reserve the right to file a continuation or divisional application on subject matter canceled by way of this Amendment.

Formal Matters

The objection to the specification has been maintained because the specification allegedly contains embedded hyperlinks on page 29. The objection is respectfully traversed.

Having previously consulted with the Patent Help Desk of the Office, Applicants' undersigned representative understands that the purpose of the prohibition of hypertext in the specification set forth in MPEP § 608.01(a) is to prevent executable code or hyperlinks from becoming active in electronically published patents on the USPTO web site. MPEP § 608.01 provides examples of text that, if present, may comprise objectionable hypertext code. MPEP § 608.01 indicates that "Examples of a hyperlink or a browser-executable code are a URL placed between these symbols "< >" and http:// followed by a URL address." *See*, M.P.E.P § 608.01(a). In conformance with the requirements of MPEP § 608.01, the paragraph on page 29 that formerly contained http prefixes was amended to delete each occurrence of "http:\\" preceding each internet address.

Applicants' undersigned representative has been informed that internet addresses, by themselves, are not objectionable hyperlinks or executable code. The specification was amended in the Amendment and Reply filed on September 2, 2003 to leave only plain internet addresses. In view of the foregoing, withdrawal of the objection is again respectfully requested.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 17 and 18 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for the recitation of "two or more different said compounds." Claim 17 previously recited "A composition comprising a polypeptide of claim 1, an analogue thereof or a combination of two or more different said compounds." Claim 1 describes a genus of polypeptides. Thus, one of skill in the art would understand that the phrase "two or more

different said compounds" of claim 17 refers to compositions comprising one of the polypeptides described by claim 1 and an analogue of a polypeptide of claim 1, two or more different polypeptides described by claim 1, and/or one or more analogues thereof.

However, simply in order to expedite the prosecution of the application and to reduce the number of issues on appeal, should an appeal become necessary, Claim 17 has been amended to recite the claimed subject matter in another way. One of skill in the art would clearly understand the metes and bounds of claim 17, as amended, and claim 18. Accordingly, withdrawal of the rejection of claims 17 and 18 under 35 U.S.C. § 112, second paragraph, is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph (Enablement)

Claims 1-3, 17, 18, 20-22 and 37 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly not enabled by the specification for the full scope of the claims. The rejection is respectfully traversed.

The specification is enabling for the full scope of the claimed invention.

The Examiner has acknowledged that the specification is enabling for a polypeptide consisting of SEQ ID NOS: 3 to 33, 65 and 66, which are exemplary "epitope" sequences described in the specification. The specification teaches, as an example, that a polypeptide of the invention can have the sequence of an exemplified epitope. See, for example, Specification at page 6. Claim 1, as amended, recites a polypeptide comprising at least one amino acid sequence of at most 20 and at least 8 consecutive amino acids defined in SEQ ID NO: 1, said polypeptide binding at least one MHC-I glycoprotein, with the proviso that said

polypeptide is different from SEQ ID NO: 2. The exemplified epitope sequences are representative of the genus of polypeptides encompassed by claim 1. This is demonstrated by claims 2 and 3, which recite that the amino acid sequence of claim 1 is selected from among the exemplified sequences disclosed in SEQ ID NOS: 3 to 33, 65 and 66.

The specification further teaches that a polypeptide of the invention can comprise one or more copies of one or more epitopes." Id. Thus, myriad polypeptides representative of the genus are disclosed and enabled in the specification by combining one or more copies of one or more of the exemplary epitopes in a polypeptide of the invention according to explicit instructions in the specification. The enablement of polypeptides containing a combination of epitopes binding at least one MHC-I glycoprotein is evidenced, for example, by Whitton et al. (*J. of Virology*, 67:348-52, 1993), cited at page 3 of the specification. (Attached as Exhibit A)

Myriad representative members of the claimed genus are disclosed by direct application of the teaching in the specification to the exemplified sequences. For example, at least 284,273 representative members of the genus are disclosed simply in considering combinations of 1, 2, 3, 4 or 5 different epitopes chosen from among the disclosed example epitopes as described in the specification at pages 6-7 and 13-15 (disclosing that a polypeptide of the invention can comprise one or more copies of one or more epitopes, optionally separated by linker sequences or protease cleavage sites.) The number of representative members of the genus is multiplied when considering that polypeptides comprising one or more copies of each epitope are also described and enabled.

The number of enabled species taught by the specification is even more enormous considering polypeptides of the invention derived by optionally including linker sequences that may be encoded, for example, by a nucleotide sequence comprising a restriction enzyme sequence or intervening protease cleavage sequences. The use of linker and protease cleavage sequences in recombinant polypeptides is enabled by the knowledge in the art.

As further examples, the specification teaches that the polypeptide can also contain non-epitope sequences, such as adjuvant and targeting sequences, and may be incorporated into fusion peptides comprising sequences from different proteins. (See, the specification at pages 3 and 7.)

The specification satisfies the requirements set forth in In re *Wands*.

The standard under which enablement is to be determined is whether, given the teaching of the specification, the state of the art, and the expectations of the skilled practitioner, it would require undue experimentation to make and use a polypeptide within the scope of the invention. *See, e.g.*, *In re Wands*, 8 U.S.P.Q. 2d, 1400 (Fed. Cir. 1988); M.P.E.P. § 2164.08. Applicants respectfully submit that the guidance provided in the specification, taken together with the knowledge in the art, is more than sufficient to guide the skilled practitioner to make and use polypeptides within the scope of the claim without undue experimentation.

The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. *In re Wands* at 1404. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the

specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *Id.* The Federal Circuit set forth factors that demonstrate enablement, (a) considerable guidance in the specification, (b) working examples, and (c) the expectation of practitioners in the field concerning the need for screening. *See, In re Wands,* at 1406.

The present claims are enabled by the specification when the *Wands* factors are considered. (a) ***Guidance***. The present specification provides significant guidance concerning the selection of amino acid sequences. For example, at page 10, the specification provides guidance with respect to preferably conserved HLA binding motifs and allowable substitutions in analogues. The specification provides example epitopes and discloses that the epitopes of the invention can be combined, optionally with linker and other functional elements, to create a vast number of representative polypeptides of the invention. (b) **Working Examples**. The specification provides working examples of 33 exemplary "epitope" amino acid sequences and provides working examples of a screening method to identify suitable polypeptides within the claimed genus. See, for example, the Examples of the specification. (c) **Reasonable Experimentation.** As in *Wands*, any screening can be done using the methods taught in the specification or other art recognized methods guided by the considerable teaching of the specification that guides selection of epitopes and construction of the claimed polypeptides.

The alleged reasons for finding that the claims are not enabled are contradicted by evidence.

For example, it is alleged in the rejection that the length of the polypeptide is important for binding to MHC-I glycoprotein. However, Whitton et al. provide evidence that epitopes can be fused into a "string of beads" and that such a string of epitopes can bind MHC-I glycoprotein. The extended length and proximity of two fused epitopes did not interfere with recognition of either epitope. *See*, Whitton et al., *supra*. Further, Engelhard, *Curr. Opin. Immunol.* 6:13-23 (1994), cited in the Official Action, teaches that longer peptides have been shown to bind with affinities comparable to those of predominant 8-mer and 9-mer sequences. (*See,* Engelhard at page 14, column 1.)

It is alleged that there are conserved residues that participate in important interactions for holding a peptide in the binding groove of MHC-I glycoprotein. It is also alleged that "The specification does not teach which changes in amino acid sequence of 7 to 20 sequential amino acids would not alter all the activities of the of the peptide." However, the specification provides extensive guidance, for example at page 10, describing acceptable substitutions in analogues and identifies conserved residues at positions 2, 6 and 9 in epitope sequences to enable the selection of amino-acid sequences according to the invention. Further, the teaching of Guo et al. (Nature, 360:364-66, 1992) that is cited in the Official Action as evidence that one of skill in the art would have been aware of conserved residues in MHC-I binding polypeptides is among the knowledge that would have been available to guide one of skill in the art in selecting amino acid sequences.

Finally, citing page 2 of the specification, it is alleged that "it is impossible to predict which fragments T cell will recognize." However, Applicants point out that the teaching of the specification provides substantial guidance and a large number of examples; so that taken with the knowledge in the field, one of skill in the art would be able to make and use functional polypeptides according to the invention.

Applicants respectfully submit that the specification enables one of skill to make and use any polypeptide of the genus described by claim 1. Myriad representative examples are disclosed in individual and combinations of the example epitopes, optionally in combination with well known non-epitope elements, according to the explicit instructions in the specification. The vast number of representative examples must be considered together with the general guidance of the specification and knowledge of one of skill in the art.

The rejection discounts the evidence of enablement of vaccine compositions according to the invention which is presented in Example 6. However, in Example 6, vaccine compositions comprising polypeptides that had been identified according to the methods taught by the specification as described in claim 1 demonstrated a protective effect in an art accepted mouse model of tumor growth. Moreover, there is evidence of enablement in the art. For example, Whitton et al., *supra*, provides further evidence that a vaccine comprising a MHC-I binding polypeptide comprising an epitope or a string of epitopes can confer a protective immune response. Thus, one of skill in the art is provided with guidance, a demonstration of how to make and use a vaccine as claimed, and evidence of its effectiveness in an art accepted model. Moreover, with respect to claims 21-22, directed to a vaccine composition, even if some screening might be required, the rejection has not provided an

explanation of why this would be considered undue in the art. That some experimentation is necessary does not preclude enablement. *See, e.g. Wands, supra.* The rejection has not shown what level of screening would be considered undue by practitioners in the field.

For at least the foregoing reasons, Applicants respectfully request withdrawal of the rejection of claims 1-3, 17, 18, 20-22 and 37 under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. § 112, first paragraph (Written Description)

Claims 1-3, 17, 18, 20-22 and 37 stand rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention at the time the application was filed. The rejection is respectfully traversed.

Claim 1, as amended, recites a polypeptide comprising at least one amino acid sequence of at most 20 and at least 8 consecutive amino acids defined in SEQ ID NO: 1, said polypeptide binding at least one MHC-I glycoprotein, with the proviso that said polypeptide is different from SEQ ID NO: 2. This genus is more than sufficiently described in the present specification.

The rejection refers to *Fiers v. Revel*, 25 U.S.P.Q. 1601, 1606 (Fed. Cir. 1993), in asserting that the written description provided by the inventors is inadequate. However, contrary to the situation in *Fiers*, in the instant case the specification provides explicit written description of many representative species of the claimed genus and defines the genus by distinguishing structural and chemical properties.

The claimed genus is described by the essential structural features of the claimed polypeptide. The polypeptide comprises at least one amino acid sequence of at least 8 and no more than 20 consecutive amino-acids defined by SEQ ID NO: 1. Additional optional structural features are further described in the specification, for example multiple copies of the epitope sequence and/or multiple different epitope sequences optionally connected by linker sequences and/or including non-epitope sequences as described in the specification. The genus is further defined by a chemical property related to these structural features which further defines the claimed genus, being a polypeptide binding at least one MHC-I glycoprotein. Structural features correlating with the recited function are described in the specification. Further knowledge concerning structures related to the functional properties of the claimed polypetides was available in the art as evidenced by publications referenced in the specification and cited in the Official Action.

It is acknowledged that applicants are in possession of polypeptides consisting of SEQ ID NOS: 3-33 and 65-66, but not the genus of polypeptides described by claim 1. Polypeptides consisting of SEQ ID NOS: 3-33 and 65-66 comprise working examples of actual reduction to practice of polypeptides of claim 1.

Actual reduction to practice is sufficient, but not necessary to demonstrate possession. Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶1 "Written Description" Requirement, 66 FR 1099, 1106 (2001). Possession can also be demonstrated by disclosure of sufficiently detailed, relevant identifying characteristics, i.e. complete or partial structure, other physical or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure.

The specification contains substantial description of component structures, chemical properties, and functional characteristics coupled with a known and disclosed correlation between structure and function. The amino acid sequences SEQ ID NOS: 3-33 and 65-66 are also described in the specification as exemplary components of polypeptides comprising multiple epitope sequences. For example, polypeptides comprising one or more of the amino acid sequences defined by SEQ ID NOS: 3-33 and 65-66, optionally connected by linker sequences or protease cleavage sites and/or connected to other non-epitopic sequences. See pages 6-8 and 13-15 of the specification. Functional characteristics coupled with a known and disclosed correlation between structure and function are recited in claim 1 and described throughout the specification. For example, at page 10, preferred conserved residues in epitope sequences are described. The specification references additional knowledge in the art that correlates the structure of epitope sequences with the recited MHC-I binding function.

Protease cleavage sequences and other suitable linker sequences that may be included in polypeptides of claim 1 are known in the art. Use of known components, in a manner auxiliary to the invention must have a written description only so specific as to lead one of ordinary skill in the art to that class of material. A functional recitation of those compounds may be sufficient. *See In re Herschler*, 200 U.S.P.Q. 711, 714 (C.C.P.A. 1979).

Possession can be shown by describing a representative number of species. *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

> A description of a genus of [biological molecules] may be achieved by means of a recitation of a representative number of [biological molecules], defined by ... sequence, falling within the scope of the genus or of a recitation of structural

> features common to the members of the genus, which features constitute a substantial portion of the genus. This is analogous to enablement of a genus under Section 112, Para. 1, by showing the enablement of a representative number of species within the genus. *See,* [*In re Angstadt and Griffin,* 190 USPQ 214, 218 (CCPA 1976)] (deciding that applicants "are *not* required to disclose *every* species encompassed by their claims even in an unpredictable art" and that the disclosure of forty working examples sufficiently described subject matter of claims directed to a generic process); *In re Robins*, 166 USPQ 552, 555 (CCPA 1970) ("Mention of representative compounds encompassed by generic claim language clearly is not required by Section 112 or any other provision of the statute. But, where no explicit description of a generic invention is to be found in the specification . . . mention of representative compounds may provide an implicit description upon which to base generic claim language.") *Id.* (emphasis added)

The number of representative species of the genus of claim 1 disclosed by the detailed description of the genus of polypeptides according to claim 1 in the specification is astronomical considering the number of polypeptides described by combinations of epitope sequences. As a non-limiting illustration, the number of disclosed and enabled polypeptides comprising 1, 2, 3, 4 or 5 different epitopes chosen from among the 33 exemplary sequences is at least 284,273 polypeptides. Many more representatives of the genus can be derived by application of the directions of the specification. The disclosure is not limited to this example. The essential structural features of each such member of the genus is described by the teachings of the specification.

In view of the at least the foregoing, the requirements of 35 U.S.C. § 112, first paragraph are met by the specification for all pending claims of the application. Accordingly, withdrawal of the rejection of claims 1-3, 17, 18, 20-22 and 37 under 35 U.S.C. § 112, first paragraph is appropriate and is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph (New Matter)

Claims 1-3, 17, 18, 20-22 and 37 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the Specification. The rejection is respectfully traversed. Without acceding to the reasons for the rejection, but simply in order to expedite prosecution and to reduce the number of issues on appeal, claim 1 has been amended to recite "A polypeptide comprising at least one amino acid sequence of at most 20 and at least 8 consecutive amino acids defined in SEQ ID NO: 1" The length of 8 amino acids as a typical lower limit for the size of the claimed polypeptide is explicitly supported in the paragraph bridging pages 6 and 7.

Rejections under 35 U.S.C. § 102

Claims 1, 17, 18, 20-22 s were previously rejected under 35 U.S.C. § 102 as allegedly anticipated by Van Baalen et al. (WO 98/17309) as evidenced by Rammensee et al. (*Immunogenetics*, 41:178-228, 1995). The rejection has been withdrawn.

Claims 1-3, 17, 18 and 20-22 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Wreschner (WO 96/03502 A2) as evidenced by Rammensee et al. (*Immunogenetics*, 41:178-228, 1995). It was alleged that Wreschner discloses a polypeptide (noted in the Official Action as SEQ ID NO: 17 although this identifier was not found in the reference) that is 100% identical to elected species SEQ ID NO: 26. It was further alleged that Wreschner discloses compositions and vaccines comprising this polypeptide.

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal*

Bros. v. Union Oil Co. of California, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 +(Fed. Cir. 1987). The elements must be arranged as required by the claim. *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990).

The Examine alleges that since the word "comprising" is considered open language, the claims read on polypeptides taught by Wreschner. However, claim 1 recites a limitation within the elements comprising the claimed polypeptide that excludes the polypeptides of Wreschner. Claim 1 recites "a polypeptide comprising at least one amino acid sequence of at most 20 and at least 8 consecutive amino acids defined in SEQ ID NO: 1 . . .." Thus, while addition elements are permitted in the amino acid sequence, claim 1 requires a sequence having at most 20 consecutive amino acids defined in SEQ ID NO: 1. None of the polypeptides taught by Wreschner comprise an amino acid sequence having at most twenty consecutive amino acid residues of SEQ ID NO: 1. There is no suggestion in Wreschner to modify a polypeptide to arrive at the polypeptides of the invention. Each of claims 1-3, 17, 18 and 20-22 incorporate the limitations of claim 1.

Wreschner does not teach or suggest all the elements of the claims, and therefore does not anticipate claims 1, 17, 18, 20-22. Accordingly, withdrawal of the rejection of these claims under 35 U.S.C. § 102(b) is respectfully requested.

Rejection under 35 U.S.C. § 103

Claim 37 stands rejected under 35 U.S.C. § 103 as allegedly unpatentable over Wreschner (supra) or Van Baalen et al. (supra) in view of Zuk et al. (U.S. Patent No. 4,281,061). The rejection is traversed.

As acknowledged in the Official Action, Wreschner does not teach or suggest a kit comprising a polypeptide and an adjuvant. Further, as described above, Wreschner does not teach or suggest a polypeptide as described in claim 1, as amended.

Zuk et al. does not teach or suggest a polypeptide as described in claim 1. Even if Zuk et al. can be read as suggesting making a pharmaceutical kit, Zuk et al. fails to cure the deficiencies of Wreschner. Therefore, because the references in combination do not teach or suggest every element of the claimed invention, a prima facie case of obviousness has not been established. Accordingly, withdrawal of the rejection is requested.

New Claims

By the present amendment, claims 38 to 56 are added. Claims 39 to 56 are dependent on new claim 38, which recites "A polypeptide consisting essentially of one or more amino acid sequences selected from the group consisting of SEQ ID NOS: 3 to 33, SEQ ID NO: 65 and SEQ ID NO: 66, wherein the polypeptide has at most 20 consecutive amino acids defined in SEQ ID NO: 1." Claim 38 recites "consisting essentially of" to indicate that the claimed polypeptide may contain one or more copies of one or more of the recited sequences in addition to elements that do not materially affect the basic and novel characteristics of the claimed invention as described in the specification. For example the polypeptides of claims 38-56 may contain linker sequences connecting the recited amino acids and may be fused to non-epitope sequences as described in the specification. No prior art of record teaches or suggests a polypeptide as recited in new claims 38-56.

Linking Claims

Group 25, comprising claims 1-3, 17-18, 20-22 and 37 has been elected for examination purposes. Claims 1, 17-18, 20-22 and 37 continue to be generic to Groups 2-34, set forth in Paper No. 19. Newly presented claims 38-56 read on the group elected for examination and are likewise generic to Groups 2-34. No prior art of record anticipates or renders the elected species or the generic claims obvious. Accordingly, Applicants respectfully request that examination encompass the entire genus described by claim 1 pursuant to M.P.E.P. § 809. The restriction requirement was timely traversed. Applicants reserve the right to petition the restriction requirement under 37 C.F.R. § 1.144.

CONCLUSION

In view of the foregoing, further and favorable action in the form of a Notice of Allowance is believed to be next in order. Such action is earnestly solicited.

In the event that there are any questions relating to this application, it would be appreciated if the Examiner would telephone the undersigned concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

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EXHIBIT A

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A "String-of-Beads" Vaccine, Comprising Linked Minigenes, Confers Protection from Lethal-Dose Virus Challenge†

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We have previously demonstrated that induction of antiviral cytotoxic T lymphocytes (CTL), in the absence of antiviral antibodies, can confer protection against a lethal-dose virus challenge. Here we extend those findings as follows. First, three discrete viral CTL epitopes expressed from minigenes encoding peptides as short as 12 amino acids can be recognized when expressed from recombinant vaccinia virus; second, concentrating on two of the three epitopes, we show that these vaccinia virus recombinants can confer protection in a major histocompatibility complex (MHC)-restricted manner; third, the minigenes can be fused to generate a "string of beads," and the close proximity of the two epitopes within one oligopeptide does not disrupt recognition of either epitope; fourth, this string-of-beads vaccine, in contrast to the single epitope vaccines, can protect on both MHC backgrounds; and, fifth, CTL to different epitopes may act synergistically, as protection is improved when the vaccine contains more than one CTL epitope for a given MHC background.

Adaptive immunity to virus infection can be conferred in either of two ways: passive, usually by transfer of immune serum, or active, by exposure to antigen. The latter method, most commonly by using as antigen live, attenuated viruses, has yielded outstanding results: smallpox has been eradicated, and the incidence of polio, measles, mumps, and rubella, among others, has declined dramatically, at least in the developed countries. Nevertheless, virus infection remains a major and, particularly since the emergence of human immunodeficiency virus, an increasing cause of human morbidity and mortality. Thus, a detailed understanding of effective vaccine responses is essential, to minimize vaccine failures and to develop new-generatation vaccines to cope with novel challenges.

The antigen-specific immune response can be divided into two arms: antibody and T lymphocyte. Antibodies recognize free antigen, while T cells recognize antigen in the form of processed peptide, bound by and presented in the groove of a host glycoprotein encoded by the major histocompatibility complex (MHC). T cells submit to subdivision into two functional groups, characterized by surface marker phenotype and by the MHC class with which they interact (for a review, see reference 2). In general, cytotoxic T lymphocytes (CTL) carry the CD8 molecule and interact with (and kill) cells expressing antigen presented by class I MHC molecules, while helper T cells, which provide help for antibody production, bear the CD4 molecule and interact with cells expressing antigen plus class II MHC molecules. Class I MHC proteins are expressed on almost all somatic cells, neurons being an exception (16), and present peptides of 9 to 10 amino acids (6, 7, 14, 28) which usually are generated from a protein made within the cell. Thus, following virus infection, fragments from even the earliest virus proteins (which generally are not cell surface proteins) can be picked up by class I molecules and displayed at the cell surface. In this way almost any somatic cell can signal that it is infected very soon postinfection. In contrast, recognition by antibody of a virus-infected cell requires expression of virus protein on the cell membrane, often a late event just preceding virus release.

Hence, when controlling primary virus infection, the potential benefit to the host of the early recognition afforded by the class I MHC-CD8$^+$ CTL interaction is clear; studies in many animal model systems have demonstrated the critical role played by CTL in eradication of primary virus infections (for a review, see reference 22). However, the role of CTL in acquired immunity (i.e., postimmunization) is less well appreciated. Antibodies are often considered the major protective components induced by vaccination; certainly, virus-specific antibody responses are induced by all of the vaccines in current use and are easily detected by routine laboratory assays. However, "experiments of nature" suggest that T cells alone play a critical role not only in controlling primary virus infection but also in protecting against disease from subsequent reexposure. For instance, congenitally agammaglobulinemic children cope well with almost all viral infections; upon measles virus challenge, they get typical disease, clear the virus, and are resistant to disease upon reexposure to measles virus, although at no time do they produce detectable antiviral antibody (9).

We have begun to dissect the importance of this antiviral acquired CTL immunity by using as a model system the arenavirus lymphocytic choriomeningitis virus (LCMV) and have shown that induction of an LCMV-specific CTL response (induced in the absence of a detectable antiviral antibody response) can protect mice from subsequent lethal-dose LCMV challenge (20, 21, 34). These findings have been confirmed in other laboratories (12, 29). This CTL immunity can be conferred by a single inoculation of a recombinant vaccinia virus expressing an LCMV protein. The effect is dependent on the MHC haplotype of the mouse and requires a class I molecule capable of binding and presenting an antigenic peptide from the LCMV protein (21). However, a recombinant vaccine expressing a single LCMV protein could protect only some mouse strains, and other strains, whose class I MHC molecules were unable to present peptides from the encoded LCMV protein, remained unpro-

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tected and succumbed to lethal-dose LCMV challenge. This problem could be overcome by expressing all viral proteins in the recombinant vector. However, for viral pathogens with large genomes, another problem would ensue; viral vectors have a limited capacity for foreign sequences, which would be rapidly exceeded if multiple complete genes were to be incorporated. In part to solve this problem, we have attempted to compress the foreign sequences by first identifying the epitopes recognized on several MHC backgrounds and by then expressing these as short open reading frames, or "minigenes," in isolation from their immunologically irrelevant protein backbone. Using expression vectors encoding minigenes designed to express peptides as short as 15 to 20 amino acids, we (35) and others (10) have previously shown that short polypeptide products can be synthesized and presented on the cell surface in a manner recognizable to T cells. The ability of these molecules to induce CTL, and the biological activity of such cells, was not analyzed. Here we extend these observations to show that first, three discrete LCMV epitopes expressed from minigenes encoding peptides as short as 12 amino acids can be recognized when expressed from recombinant vaccinia virus; second, concentrating on two of the three epitopes, we show that these vaccinia virus recombinants can confer protection in an MHC-restricted manner; third, the minigenes can be fused to generate a "string of beads," and the close proximity of the two epitopes within one oligopeptide does not disrupt recognition of either epitope; fourth, this string-of-beads vaccine, in contrast to the single epitope vaccines, can protect on both MHC backgrounds; and, fifth, CTL to different epitopes may act synergistically, as protection is improved when the vaccine contains more than one CTL epitope for a given MHC background.

MATERIALS AND METHODS

Mouse strains used. Mouse strains [C57BL/6 ($H2^{bb}$) and BALB/c ($H2^{dd}$)] were obtained from the breeding colony at Scripps Research Institute. Mice were used at 6 to 12 weeks of age.

Cell lines and viruses. BALB C17 ($H2^{d}$) and MC57 ($H2^{b}$) cell lines are maintained in continuous culture in the laboratory. Both lines are maintained in RPMI supplemented with 7% fetal calf serum, L-glutamine and penicillin-streptomycin. Viruses used were LCMV (Armstrong strain) and recombinant vaccinia viruses as outlined below.

Construction of recombinant vaccinia viruses. Recombinant vaccinia viruses carrying minigenes were constructed as follows: minigene sequences were designed and synthesized as complementary synthetic oligonucleotides, and the double-stranded DNA was cloned into a vaccinia virus transfer vector and subsequently introduced into the virus by homologous recombination, as described previously (36). In all cases, the recombinant plasmids were sequenced to ensure the correct sequence and orientation of the minigenes. Furthermore, to ensure that recombination had occurred faithfully, the appropriate region of the recombinant vaccinia virus DNA was amplified by polymerase chain reaction (PCR), and the resulting DNA was sequenced.

Cloning procedures and DNA sequencing. All cloning procedures were carried out according to the manufacturer's recommendations, and DNA sequencing was done using the dideoxy chain termination method with double-stranded template DNA and Sequenase version 2 (U.S. Biochemical Corporation, Cleveland, Ohio).

In vitro cytotoxicity assays. These assays were carried out as previously described (36). Effector cells were either day 7 primary splenocytes or were CTL clones of previously defined specificity. Target cells were infected appropriately with virus, labeled with ^{51}Cr, washed, and incubated for 5 h with effector cells at the indicated effector-to-target ratio. Supernate was harvested, and specific Cr release was calculated by using the following formula: [(sample release − spontaneous release) × 100]/(total release − spontaneous release).

A

Recombinant & Presenting MHC	DNA sequence & epitope peptide sequence
MG3 $GP1/D^b$	AGGATGAAGGCTGTCTACAATTTGCCACCTGTGGGGTAACTAGTTAA M K A V Y N F A T C G V T S -
MG4 NP/L^d	ACCATGGAAAGGCCCCAGGCTTCAGGGGTATATATGGGGAACTTAACAGTAACTAGTTAA M E R P Q A S G V Y M G N L T V T S -
MG7 $GP2/D^b$	GCCATGAGTGGGGTGGAGAATCCAGGTGGTTATTGCCTGTAA M S G V E N P G G Y C L -
MG34 $GP1/D^b$ + NP/L^d	CCCATGAAGGCTGTCTACAATTTGCCACCTGTGGGGGGAGGACCATGGAAAGGCCCCAG M K A V Y N F A T C G G R T M E R P Q GCTTCAGGGGTATATATGGGGAACTTAACAGTAACTAGTTAA A S G V Y M G N L T V T S -

B

Numbering for 9-residue motif		1 2 3 4 5 6 7 8 9
Db consensus motif (6), with proposed functions in next column	Anchor	N M
	Strong	M I K L I L E F P Q V V
LCMV GP1 in MG3	matches with	M K A V Y N F A T C G V T S
LCMV GP2 in MG7	motif are in bold	M S G V E N P G G Y C L

FIG. 1. The DNA sequences of the minigenes and the encoded amino acids. (A) DNA and encoded peptide sequences of the four constructs described in the text. The LCMV sequences are shown in boldface, and non-LCMV sequences are in normal type. (B) Consensus nonameric peptide motif for the D^b class I MHC molecule, the proposed role of each residue, and the location of matching residues (shown in boldface) in the two LCMV epitopes presented by D^b.

RESULTS

Construction of recombinant vaccinia viruses encoding minigenes. Figure 1 shows the DNA and amino acid sequences of the minigenes which were studied. The minigenes from the two mature glycoproteins GP1 and GP2 of LCMV (MG3 and MG7, respectively) are presented by the D^b molecule while that from the LCMV nucleoprotein (NP) (MG4) is presented by the L^d molecule. Finally, two of the minigenes (MG3 and MG4) were linked in tandem (connected by three nonviral amino acids) in construct VVMG34. All DNA sequences were confirmed both in the vaccinia virus transfer plasmid and directly from a polymerase chain reaction product of vaccinia virus DNA. In all cases, the ATG shown is the first one in the RNA transcript. Also shown in Fig. 1 are the peptide consensus motif for binding and presentation by the D^b molecule (6, 27) and the relative alignments of the two LCMV minigenes presented by this molecule (no such motif has been obtained for the L^d molecule).

Recognition of minigenes by primary CTL and by CTL clones. VVMG3, VVMG4, VVMG7, and VVMG34 were used to infect $H2^b$ or $H2^d$ target cells which were subsequently incubated with the appropriate effector cells, as shown in Table 1. As can be seen, VVMG3 is recognized by

In vivo protection studies. Mice (6 to 12 weeks of age) were inoculated with a single intraperitoneal (i.p.) dose of 2×10^7 PFU of recombinant vaccinia virus, a single dose of LCMV Armstrong (2×10^5 PFU i.p.) as a positive control, or with 200 μl of medium i.p. as a negative control. Six weeks later, the animals were challenged with a potentially lethal dose (20 50% lethal doses) of LCMV administered intracranially. Mice were observed daily, and all recorded deaths occurred between days 6 and 12 following lethal-dose LCMV challenge.

TABLE 1. Recognition of single epitopes and a string-of-beads construct on two MHC backgrounds[a]

Effectors	E:T[c]	% Specific Cr release[b] from following target cells:													
		$H2^{bb}$ (MC57)							$H2^{dd}$ (BALB C17)						
		UN	LCM	SC11	MG3	MG4	MG7	MG34	UN	LCM	SC11	MG3	MG4	MG7	MG34
$H2^{bb}$	50	6	**40**	3	**49**	6	**11**	**42**							
Splenocytes	25	4	**30**	1	**37**	5	**6**	**30**							
$H2^{dd}$	50								0	**68**	0	**1**	**65**	0	**60**
Splenocytes	25								1	**61**	0	0	**54**	0	**50**
αGP1/D^b	5	0	**40**	0	**37**		0	**35**							
αGP2/D^b															
	5	0	**40**	0	0		**60**	0							
	1	0	**16**	0	0			0							
αNP/L^d	5							0	**38**	0	0	**30**	0	**35**	

[a] In vitro cytotoxicity assays were carried out as described in Materials and Methods. Effector cells were primary splenocytes (taken 7 days post-LCMV infection) from $H2^{bb}$ or $H2^{dd}$ mice or cloned cell lines of known epitope specificity and MHC restriction.
[b] Levels above background are in boldface.
[c] E:T, effector-to-target cell ratio.

$H2^b$ primary CTL and by $H2^b$ CTL clones specific for the GP1 epitope but is not recognized by any $H2^d$-derived CTL. VVMG7 is recognized by $H2^b$ primary spenocytes (at a low level, characteristic of this epitope [35]) and directs a high level of lysis by an anti-GP2 CTL clone. Conversely, VVMG4 is recognized by $H2^d$ primary CTL and $H2^d$ anti-NP CTL clones but not by $H2^b$ cells. Thus, each of these individual minigenes produces a short peptide capable of being correctly processed and presented by class I MHC. When the $H2^b$/GP1 and $H2^d$/NP epitopes are linked together in VVMG34, the recombinant virus is recognized both by $H2^b$ and $H2^d$ primary CTL and by the appropriate CTL clones (Table 1). Thus, the close physical linkage of these two epitopes does not prevent their correct processing and presentation by different class I MHC molecules.

Minigenes: protection studies and a string-of-beads vaccine. VVMG3, VVMG4, and VVMG34 along with the control virus VVSC11 were used in vaccination studies. Which of these agents would protect various mice of various MHC haplotypes? As can be seen from Fig. 2, VVMG3 containing the $H2^b$ GP1 epitope protected 85% of $H2^b$ mice. Thus, this minigene vaccine, which is recognizable by CTL, is capable also of inducing protective responses. Note also that this recombinant virus conferred some protection (38%) on $H2^d$ mice. This was unexpected, as primary $H2^d$ CTL and over 30 $H2^d$ CTL clones which we derived all were directed towards the single NP epitope and did not recognize $H2^d$ target cells infected with VVMG3. A single $H2^d$ CTL clone which recognized the virus glycoprotein (unpublished data; clone no longer available) was obtained, and initial mapping showed recognition of a CTL epitope on the $H2^d$ background which lay between GP residues 1 to 60. Thus, it appears likely that this epitope is present in VVMG3; we have previously reported epitopes shared between several MHC backgrounds (24). VVMG4, as expected, protects $H2^d$ mice (94% survival) but not $H2^b$ mice (no survivors). VVMG34 protects 100% of $H2^{dd}$ mice and 87% of $H2^{bb}$. Therefore, two CTL epitopes in close proximity do not prevent each other from inducing appropriate responses.

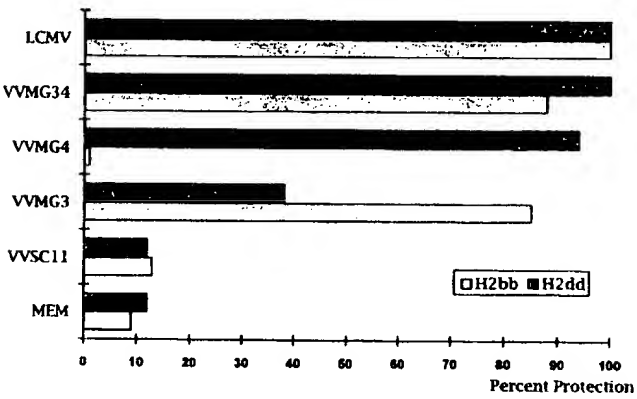

FIG. 2. Protection against lethal-dose LCMV challenge. Mice (BALB/c $H2^{dd}$ or C57BL/6 $H2^{bb}$) were vaccinated as described in the text, with the materials shown on the y axis, and 6 weeks later were challenged with a normally lethal intracranial dose of LCMV. Mice were observed daily for 21 days. All deaths occurred within 12 days of challenge.

DISCUSSION

Here we confirm and extend previous observations by using minigenes to study antiviral CTL responses. We make three novel observations. First, CTL epitopes as short as 12 amino acids (VVMG7, 11 LCMV residues linked to a Met initiator) can, when encoded in recombinant vaccinia viruses as minigenes, be recognized by virus-specific CTL. Second, a minigene encoding 14 residues (10 viral and 4 nonviral) can confer protection against lethal-dose viral challenge when administered as a single dose of recombinant vaccinia virus. By using VVMG3 or VVMG4, good protection is conferred upon the appropriate MHC haplotypes, but less or no protection is seen in mice of other MHC backgrounds. Third, multiple short minigenes can be arranged in tandem; to improve vaccine coverage and efficacy, we expressed in VVMG34 the two minigenes in tandem, in a string-of-beads arrangement. Despite their close proximity in this construct, both epitopes were recognized by appropriate CTL, and a protective response was elicited in both $H2^{bb}$ and $H2^{dd}$ mice. All of these findings have implications for both the development of vaccines and the processing and presentation of MHC class I restricted epitopes.

The use of live, attenuated viral vaccines has contributed greatly to the diminution or eradication of several viral diseases. Our aim is to dissect the components of protective immunity induced by immunization and to design new vaccines which are both safe and effective, protecting most or all individuals from disease. Although the virus-specific antibody response is considered critical in immunization—antibody responses are often used as a surrogate measure of vaccine-induced immunity—we and others have shown that the induction of antiviral CTL, in the absence of antiviral antibody induction, can protect against challenge with a normally lethal dose of virus (12, 17, 19–21, 26, 30). The epitopes encoded in the recombinant vaccinia viruses described here do not induce detectable LCMV-specific antibody responses. Most previous studies used recombinant vaccinia viruses encoding full-length viral proteins or relatively large subfragments thereof, although in one study a short CTL epitope of murine cytomegalovirus was embedded in a much larger protein, the surface antigen of hepatitis B virus, and this molecule was shown to present the epitope in a biologically relevant manner (4). However, in order to protect an outbred population, such as humans, a vaccine must induce responses on most or all MHC backgrounds and, as shown herein, one defect in subunit vaccination is the risk of vaccine failure due to nonresponder vaccinees. Both of the single minigene recombinants are susceptible to vaccine failure; when equal numbers of $H2^{bb}$ and $H2^{dd}$ mice are vaccinated, VVMG3 protects 61% of the mixed population and VVMG4 protects only 47%. Vaccines must, therefore, contain sufficient immunogenic information to induce protective responses on most or all haplotypes; if lengthy proteins had to be used, this would necessitate cloning of lengthy DNA fragments into the expression vector. Thus, the relatively small capacity of live viral vectors for foreign DNA may argue against their use as a delivery system for immunogenic sequences; even vaccinia virus may have an upper limit of 25 kb. In part for this reason, others have assessed bacterial delivery systems, which have a much larger potential capacity. However, by using this minigene approach, it would be possible to encode around 50 immunogenic fragments in an average-size protein (50,000 Da, 1,500 bp). In the present study, vaccine failure was diminished by use of the string-of-beads vaccine VVMG34. The benefit of this recombinant to an equally mixed population, when compared with that of recombinants containing a single epitope, is clear; 93.5% of vaccinees are protected. Thus, renewed consideration can be given to live delivery systems such as viral vectors and to immunization with replication-incompetent plasmid DNA (31, 37). The results in Fig. 2 also suggest that any protective effects of individual epitopes may be synergistic. For example, both VVMG3 and VVMG4 protect a certain proportion of $H2^{dd}$ mice (38 and 94%, respectively), and their combination confers 100% protection. Moreover, VVMG3 protects 85% of $H2^{bb}$ mice, while VVMG4 confers no protection; the combined vaccine VVMG34 confers a level of protection virtually identical to that conferred by VVMG3 alone (88 and 85%, respectively). Thus, two benefits may accrue from such combinatorial immunization. First, protective responses can be elicited on several MHC backgrounds, and second, on a single background the biological effectiveness of the response may be enhanced. We have concentrated on live vaccines because most studies to date have shown that the immunity resulting from this is more solid, and of greater duration, than that induced by killed vaccines (15, 32). The alternative approach to CTL induction uses soluble polypeptides, either in the form of ISCOMs (23) or as short synthetic peptides. In the LCMV system, peptides can be injected (with incomplete Freund's adjuvant) to induce an antiviral response (1), and subsequent analysis (following peptide injection with or without adjuvant) showed protection against viral challenge (30). Similar results have been observed by using Sendai virus (18). The precise requirements for peptide-mediated induction remain unclear, although recent data suggest that such peptides contain an epitope recognized by $CD4^+$ T cells (classically, helper cells), in addition to the epitope recognized by $CD8^+$ T cells (8).

The mechanism by which immunogenic peptide fragments are presented on class I molecules on the cell surface is the subject of intense investigation. What effects do the amino acid sequences within and around the minimal epitope have on processes which occur prior to binding to the class I molecules, for instance, in the generation of immunogenic peptides and/or their transport into the endoplasmic reticulum? Evidence that at least some level of sequence specificity is present at the level of transport comes from the rat RT1a system, in which polymorphism in a putative peptide transporter leads to a different spectrum of peptides being bound by identical class I molecules (25). Furthermore, it has been suggested that certain residues flanking the peptide comprising the CTL epitope may inhibit its eventual presentation. Studies of minigene constructs of influenza virus epitopes revealed such an effect (5), and in one case, the protective efficacy of an epitope embedded in a larger protein was diminished by the flanking residues (3). In contrast, others have found little or no effect of flanking sequences (11), which is mirrored by our results. First, our observation that an epitope of only 12 amino acids can be processed and presented normally suggests that extensive flanking residues are not required for processing and presentation. Second, despite the close proximity of these two CTL epitopes, both are recognized on the appropriate MHC backgrounds, and a protective response is elicited on both $H2^b$ and $H2^d$ backgrounds. Thus, the flanking sequences in this case have little or no effect on antigen presentation or on the biological efficacy of this vaccine. Finally, it is unclear whether the peptides generated from minigenes undergo further processing within the cell prior, or indeed subsequent, to their association with class I molecules. It is known that epitopes present on signal peptides that enter the endoplasmic reticulum by routes other than the class I processing pathway appear to undergo no further proteolytic cleavage (13, 33). We are currently investigating the further processing of peptides that enter the endoplasmic reticulum through the normal pathway by expressing the minigenes described here within cell lines deficient in processing or transport machinery and by altering the minigene sequences by site-specific mutagenesis.

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